EXPLORING ACYL HOMOSERINE LACTONE AS A POSSIBLE BIOMARKER TO DETECT BACTERIAL CONTAMINATION

Synopsis

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RANU DAYAL

DR. DINESH KUMAR
Co-Supervisor
Assistaant Director (Tech.)
Food Safety and Standards Authority of India

DR. PREM SARAN TIRUMALAI
Supervisor

Department of Botany, Faculty of Science
Dayalbagh Educational Institute (Deemed to be University)
Dayalbagh, Agra – 282 005

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1.0 INTRODUCTION

1.1 The Domino Effect in Global Food Systems - From Contamination to Security

Food, one of the most fundamental human needs, has found itself at the centre of a nexus of challenges that ripple through societies and economies worldwide (FAO, 2022). A close examination reveals an intricate chain linking food contamination to spoilage, leading to wastage, inducing shortages, and ultimately, threatening food security. This essay explores this cascade of events, shedding light on the interconnectedness of these issues and their profound implications for the global community (Ishangulyyev*et al*., 2019).

At the onset of our food journey is contamination. Contamination can be microbial, chemical, or physical, introduced inadvertently during production, processing, distribution, or preparation. Harmful bacteria, viruses, parasites, or chemical substances compromise the safety of the food and pose health risks to consumers (Borchers *et al*., 2009). For instance, the contamination of crops due to the excessive use of pesticides or the introduction of pathogens during food processing can render food unsafe (WHO, 2022; Damalas and Eleftherohorins.,2009).

Contamination often acts as a precursor to spoilage. While not all contaminated food is spoiled, contamination can accelerate the spoilage process (Cook *et al*.,2009; Zhang *et al.*,2021). Spoilage, characterized by changes in the taste, smell, appearance, or texture of food, (Olatuji *et al*., 2022) is a clear indication that food is no longer fit for consumption. Microorganisms play a significant role here, breaking down food components and producing waste products, leading to the undesirable characteristics we associate with spoiled food (Ciani *et al*., 2021; Huisin't veld,1996).

Spoiled food usually ends up as waste. Food wastage is a colossal issue; globally, about a third of all food produced is wasted (Melikoglu *et al*.,2013). This translates to roughly 1.3 billion tons per year (Connor *et al*.,2022). Such wastage occurs at various stages of the food supply chain: from farms to processing units, retailers to consumers (Aung and Chang,2014). While factors like overbuying, improper storage, and cosmetic standards contribute to wastage, spoilage remains a key culprit (Kamla and Kumar, 2018; Saucier, 2016). The irony of the situation becomes evident when food wastage is juxtaposed against food shortages (Reynolds *et al*.,2020). The wasted food, if saved, could feed billions. Food shortages, often seen in regions with limited agricultural capacities or socio-economic

challenges, lead to increased food prices, malnutrition, and hunger. It's a paradoxical situation where vast amounts of food are discarded even as large sections of the population starve (Dau *et al*.,2016; Grote, 2014; Simon *et al*., 1980).

At the crux of this chain lies food security, a concept that encompasses both the availability and accessibility of food. According to the United Nation's Food and Agriculture Organization, *"Food security exists when all people, at all times, have physical and economic access to sufficient, safe, and nutritious food"*(Applanaidu *et al*., 2014).The cascade from contamination to wastage and shortage directly impinges upon food security. The wasted resources (like water, land, and energy) utilized in the production of wasted food, coupled with the economic ramifications of food shortages, further exacerbate the food security situation(Finley *et al*., 2004;Fullbrook, 2010; Pricfer*et al*.,2016).

However, it's not all grim. Recognizing these interconnections offers opportunities for intervention. Reducing contamination through safer agricultural and food processing practices can decrease spoilage. Cutting down on spoilage directly impacts wastage, and addressing wastage can alleviate shortages, ultimately strengthening food security(Quintieri *et al*.,2023).

The global food system, with its intricate web of challenges, therefore, presents both dilemmas and opportunities. To safeguard the future of food, a holistic understanding and a collaborative approach are crucial. As stakeholders in this system, it is incumbent upon us to ensure that food, in its journey from farm to fork, is treated as the precious resource it truly is. Only through such a comprehensive approach can we hope to achieve true food security for all.

1.2 Detection as Deterrence: The Imperative of Early Contamination Alert Systems

At the heart of the global food chain's vulnerabilities, as delineated in our exploration of food contaminations cascading consequences lies the pivotal issue of timely detection. Addressing contamination at its nascent stages could serve as a keystone remedy, potentially disrupting the chain leading from spoilage to food security concerns. An early, fast, and efficient means of detecting contamination is not just a luxury but an absolute necessity in our quest for global food safety and security(Magan.,2006).Every minute that contamination goes undetected in the food supply chain, the ramification and multiply (Dabbene*et al*., 2014). From affecting the health of consumers with potential outbreaks of foodborne illnesses to the economic repercussions of massive product recalls, the stakes

are incredibly high (Mclinden*et al*.,2014; Scharff.,2012). Consider, for example, a contaminated batch of lettuce. If not detected swiftly, it could lead to widespread illness, public panic, significant financial losses for producers, and the wastage of vast quantities of food that are discarded as a precaution(Todd,1985; Adely and Ryan., 2016; González &Ferrús.,2011).

The need of the hour therefore is the fusion of science and technology to devise state-of-the-art detection systems. Advancements in microbiology, molecular biology, and analytical chemistry can be harnessed to develop rapid detection techniques. Imagine sensors integrated into food packaging that change color when contamination is detected, or portable devices that offer real-time results on potential microbial contaminants. Such innovations could revolutionize the way we monitor food safety(Hall,2002;Naravaneni&Jamil., 2005;Kuswandi*et al*.,2011).

The challenge, however, is not just developing these technologies but ensuring their accessibility and affordability, especially in regions with limited resources. It's crucial to remember that contamination is not a localized issue. In our interconnected world, a contamination scare in one part of the globe can have ramifications worldwide (Schirone*et al.*,2017). International collaborations, public-private partnerships, and knowledge sharing can play a crucial role in this endeavor.

In our intricate global food system, contamination is an inevitable challenge (Brocklehurst,2004) However, its consequences, from spoilage to security concerns, can be mitigated with a proactive stance on detection. As we progress into an era marked by technological marvels, the onus is on us to leverage this expertise for the greater good, ensuring that every morsel of food on our plates is not just nourishing but also unequivocally safe.

This research study is aimed at developing a technique that will help us reduce food spoil ageand food wastage by address in the elementary concern of food contamination. This technique aims to explore the possibility of developing an unconventional method of detection of microbial contamination. A novel rapid and efficient detection method for microbial contamination using quorum sensing molecule as a biomarker and target molecule.

2.0 REVIEW OF LITERATURE

As per the 2019 report by the United Nations (UN), Department of Economic and Social Affairs, on population prospects, if the current trend of population growth rate continues, the world population is

projected to reach about 8.5 billion in 2030 and 9.7 billion by 2050 (FAO, 2019; Sadigov, 2022). In such a scenario the major problem that would arise is that of Food Security, in other words availability and accessibility of safe and wholesome food. If further probed, this directs us to2 major concerns :-

I. Shortage due to food spoilage

II. Availability of safe food

Both the concerns are grave and can be controlled by just arresting contamination of food. Food contamination results in spoilt or tainted foods that include pathogens such as bacteria or parasites or toxic compounds that make them unfit for human consumption(Kamala and kumar,2018;Sharif *et al*.,2014). Microbial contamination in food can arise from various sources, from the time food is grown or raised until it reaches the consumer. Identifying these sources is crucial to apply appropriate interventions for food spoilage and food safety(Gorny,2005;Tropea,2022).

Microbial contamination in food can arise from various environmental sources such as soil and water, both rich in microorganisms, with some being pathogenic. Floating dust and air might carry microbial spores that can land on and spoil food (Schuerger*et al.*,2018). Animals, during their processing, present another contamination risk, both from their intestinal tracts and from external sources like skin, feathers, and fur (Bryan & Doyle, 1995). Humans can introduce contaminants through unclean hands, respiratory secretions, or wounds. Equipment, if not thoroughly cleaned, can be a hub for microbial growth and lead to cross-contamination, while pests such as insects, rodents, and birds act as vectors for pathogenic microbes (CDC,2020; Salaheenet *al*., 2015;Cantrell *et al*.,2023).

Furthermore, the use of contaminated raw ingredients or additives, untreated water in processing, and improper food storage conditions can further enhance the risk (Pandey *et al*.,2014). Additionally, the deliberate inclusion of inferior or substandard materials in food, known as adulteration, can introduce further contaminants, emphasizing the need for stringent safety measures across the entire food chain.A food contaminant can be biological, chemical, or physical in nature, with the former being the more frequent (Hussain,2016) (Figure-1).Microbial contamination of food is a major concern for global public health, food security, and the economy. When food becomes contaminated with harmful bacteria, viruses, parasites, or fungal pathogens, it can lead to spoilage and foodborne diseases.

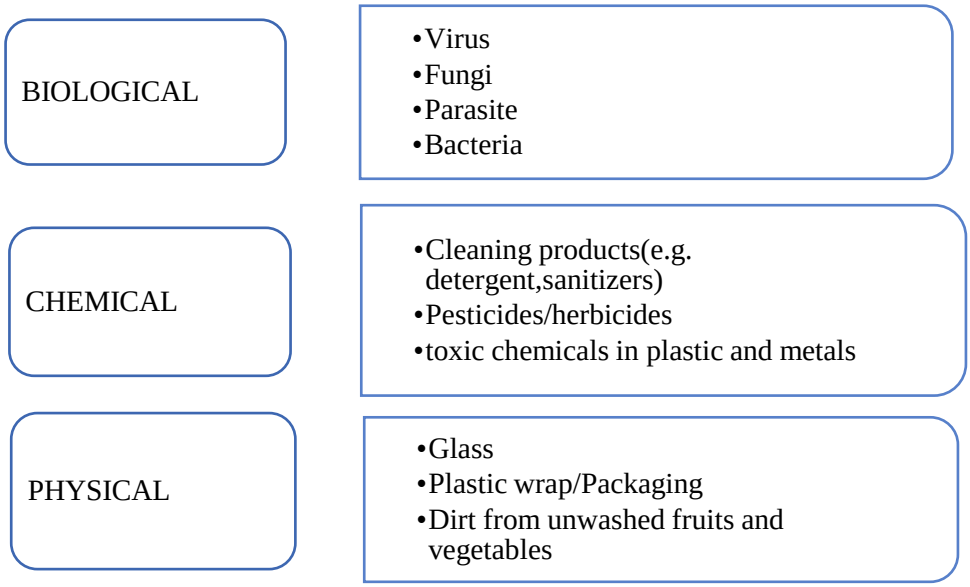

Fig 1:The type of food contamination and their cause

2.1 Shortage due to food spoilage :

The Food and Agriculture Organization (FAO) of the United Nations has frequently highlighted the significant amount of food that is lost or wasted globally. Roughly one-third of the food produced in the world for human consumption every year approximately 1.3 billion tons gets lost or wasted. The cost of the globally lost or wasted food amounts to roughly $680 billion in industrialized countries and $310 billion in developing countries.

Food spoilage is a significant contributor to food shortage and wastage worldwide.(Jood and Cai , 2016)While the underlying causes for spoilage can be varied including chemical and enzymatic reactions, it largely pertains to the growth and activity of microorganisms such as bacteria, fungi, and yeasts. Food spoilage is a process involving growth of microorganisms to numbers ranging from 10^7 to 10^9 colony forming units(CFU)/gm. Microbial spoilage of foods occurs when microorganisms, such as bacteria, fungi, and yeasts, break down food components, leading to undesirable changes in taste, smell, appearance, and texture (Lorenzo *et al*.,2018). The nature of the spoilage depends on the type of microorganism and the food substrate. Microbial spoilage of foods can be of different types, the list of the same is shown in the table below.

Table 1. Effect of microorganism on their food substrate

Type of Spoilage	Causing Microorganisms	Affected Foods	Characteristic Changes
Putrefaction	Proteolytic bacteria	Meat, fish, poultry	Off-odors, sliminess, discoloration

Fermentation	Yeasts, lactic acid bacteria	Fruits, vegetables, dairy products	Gas production, souring, texture changes
Ropiness	*Bacillus species*, lactic acid bacteria	Milk, sauces, bread	Slimy, rope-like consistency
Mold Spoilage	Filamentous fungi	Bread, fruits, jams	Fuzzy/powdery growth, off-odors, discoloration, texture changes
Acid Spoilage	Acid-producing bacteria	Milk and other foods	Sharp, sour taste
Discoloration	Pigment-producing bacteria, yeasts	Cheese, yogurt	Pink, blue, or other discolorations
Slime Formation	*Alcaligenes* species	Meat	Slimy layer on food surface
Gas Production	*Clostridium* species, some fermentative bacteria	Cheeses, packaged meats, canned goods	Swollen packages, bubbles
Oxidation of Fats	Microorganisms with lipolytic enzymes	High-fat foods	Rancidity
Alkaline Spoilage	Certain bacteria	Fish	Soapy, bitter taste due to amines and ammonia
Myotoxin Production	Certain molds like *Aspergillus*	Grains, nuts	Presence of harmful toxins, e.g., Aflatoxin in peanuts

2.2 **Availability of 'safe' food**

The World Health Organization (WHO) estimates that close to 10% of the global population becomes sick each year from consuming tainted food. This results in roughly 420,000 fatalities annually. Disturbingly, 30% of these deaths, or 125,000, involve children below 5 years of age. With around 600 million reported instances of foodborne illnesses every year, the significant public health impact of this issue becomes evident.

Similarly, according to the Centre for Disease Control and Prevention (CDC), approximately 48 million people get sick (not die) from foodborne diseases of which around 128,000 are hospitalized, and about 3,000 die each year in the United States alone. There have been several reported incidents of food borne diseases and outbreaks throughout the history that highlights the importance of confronting food contamination as a top public health priority.

These incidents, which are frequently caused by tainted food, have resulted in serious health consequences, economic losses, and, unfortunately, loss of life (Bean *et al*., 1990;Greig *et al.*,2007;Newell *et al*.,2010).

The table given below gives a general overview of some recent bacterial contamination outbreaks, globally.

Table 2 list of some recent bacterial contaminations outbreaks

Year	Location	Bacteria	Disease/Symptoms	Common Sources/Outbreak Link
2018	United States	*Escherichia coli* O157:H7	Hemorrhagicdiarrhea, Hemolytic Uremic Syndrome	Romaine lettuce from Yuma, Arizona
2019	United States	*Listeria monocytogenes*	Listeriosis	Hard-boiled eggs distributed to various retailers
2020	United Kingdom	*Salmonella*	Salmonellosis: Diarrhea, fever, abdominal cramps	Eggs
2021	Spain	*Campylobacter jejuni*	Campylobacteriosis: Diarrhea, abdominal pain, fever	Chicken product

If we were to consider the statistics of the developed nations wherein contamination of food is lower, say for instance the United States, the situation is growing to be alarmingly perilous (Akpan *et al*.,2023). Needless to mention the situation in the developing and the 3rd world nations in this regard. The tables below indicate the situation in the United States alone.

Table 3. Data of Foodborne Illness Outbreaks, Hospitalizations, and Deaths in the U.S. (1998-2008)

	1998-99	2006-08	Total	Percentage Increase
Total Outbreaks	1,856	4,912	13,405	169.40% ^
Cases of Illnesses	22,539	84,581	2,73,120	275.40% ^
Hospitalization	1,014	8,095	9,109	698.20% ^
Death	51	149	200	192.20% ^

Table 4. Outbreaks and Percentage of Affected Population by Pathogens

Pathogens	Number of outbreaks	% of people affected
Virus	3,283	41,12
Bacteria	3,613	45,25
Chemical and Toxin	685	8.59
Parasites	t67	0.84

A concise list of foodborne bacterial pathogens with ailments and food sources is shown in the table below:

Table 5 list of Common Foodborne Bacterial Pathogens, Associated Ailments, and Food Sources

Pathogen	Disease	Food Sources
Clostridiumbotulinum	Botulism	Improperly canned foods, especially home-canned vegetables, fermented fish, baked potatoes in aluminum foil
Clostridiumperfringens	Perfringens food poisoning	Meats, poultry, gravy, dried or precooked foods, time and/or temperature-abused foods

E. coli (Escherichia coli) producing toxin	*E.coli* infection (common cause of "travelers' diarrhea")	Water or food contaminated with human feces
E .coli 0157: H7	Hemorrhagic colitis or E. coli O157:H7 infection	Undercooked beef (especially hamburger),unpasteurized milk and juice, raw fruits and vegetables (e.g. sprouts), and contaminated water
Bacillus cereus	B. cereus food poisoning	Meats, stews, gravies, vanilla sauce
Staphylococcus aureus	Staphylococcal food poisoning	Unrefrigerated or improperly refrigerated meats, potato and egg salads, cream pastries
Salmonella	Salmonellosis	Eggs, poultry, meat, unpasteurized milk or juice, cheese, contaminated raw fruits and vegetables
Listeria monocytogenes	Listeriosis	Unpasteurized milk, soft cheeses made with unpasteurized milk, ready-to-eat deli meats
Vibrio vulnificus	*V. vulnificus* Infection	Undercooked or raw seafood, such as shellfish (especially oysters)
Vibrio parahaemolyticus	V. parahaemolyticus infection	Undercooked or raw seafood, such as shellfish
Shigella	Shigellosis or Bacillary dysentery	Raw produce, contaminated drinking water, uncooked foods and cooked foods that are not reheated after contact with an infected food handler

2.3 Microbial Contamination

Microbial contamination is therefore the elementary, yet prime subject of concern in controlling spoilage causing and pathogenic microbes in food. To control the contamination of food by microbes numerous traditional and modern methods are adopted (Anklam & Battaglia., 2001; Adams & Mos., 2000; Alegbeleye *et al*., 2018). A summary of the same is shown in the table below.

Table 6 list of traditional methods for controlling microbial contamination in food

Method	Description
Thermal Processing	
- Pasteurization	Mild heat treatment for liquids like milk to kill pathogenic bacteria.
- Canning	Sealing food in airtight containers followed by heating to destroy microbes.
- Blanching	Brief heat treatment to inactivate enzymes before freezing or drying.
Refrigeration and Freezing	
- Refrigeration	Storing food at low temperatures to slow microbial growth.
- Freezing	Halts microbial growth and enzyme activity.
Drying or Dehydration	Removing water to prevent microbial growth using methods like sun drying, air drying, or food dehydrators.
Modified Atmosphere Packaging (MAP)	Altering gas proportions inside packaging to inhibit microbial growth.
Preservatives	Chemical agents added to food to inhibit microbial growth, e.g., nitrates, sulfites, sorbates.

High Pressure Processing (HPP)	Subjecting food to high pressures to inactivate pathogens without heat.
Irradiation	Using ionizing radiation (gamma rays, X-rays, or electron beams) to kill pathogens and extend shelf life.
Fermentation	Transforming food using beneficial microorganisms, often creating unsuitable conditions for harmful microbes.
Salting or Sugaring	Using high concentrations of salt or sugar to inhibit microbial growth.
Vacuum Packing	Removing air to reduce oxygen and slow the growth of aerobic microorganisms.
Acidification	Lowering food pH using acids to inhibit many harmful microbes.
Osmotic Control	Using solutes like salts and sugars to reduce available water for microbial growth.
Ultraviolet (UV) Light	Disinfecting food or surfaces by exposure to UV light.
Ozonation	Using ozone gas as an antimicrobial agent for treating water and some food products.

The rate of contamination in processed foods can vary widely based on several factors, including the type of food, the processing method, the hygiene standards of the processing facility, storage conditions, and the region or country where the food is produced (Fatimah *et al*.,2011). Processed foods in developed countries generally have lower rates of contamination due to stricter regulations, better infrastructure, and advanced processing techniques. However, outbreaks and recalls do still occur. In developing countries, the risk might be higher due to challenges in infrastructure, regulation, and monitoring. An indicator of contamination in processed foods can be the frequency and cause of food recalls. In countries like the US, the FDA and USDA regularly report recalls related to microbial contamination, foreign matter presence, or undeclared allergens. A recall is an action taken by a manufacturer or distributor to remove a product from the market when it is discovered that the product may pose a health or safety risk to consumers or violates regulatory standards(Soon *et al*.,2020; Soon &Wahab., 2021).

Recalls can be initiated by the manufacturer or distributor themselves, or they can be mandated by regulatory agencies. Recalls, however are not simple as they may sound. The economic burden due to recalls are heavy, besides recalls are in response to detection of contamination in food that has already found a place in the market and recall is a measure to contain the situation(Seeks *et al*.,2021; Roberts,1998;Focker& van der Fels-Klerx, 2020).

One of the primary ways of averting large scale food spoilage or a disease outbreak is by early and

timely assessment of contamination. Despite processing and preservation, measures adopted for packaged foods, theyare due to be screened for contamination (Tao *et al*.,2014). The screening involves estimation of overall microbial count, specific type of microbe counts and detection of pathogens, all of which depends on the type of food and the type of process method the food is subject to. Foods that are processed to produce commercially sterile (95% sterile) packages are subject commercial sterility testing (Diep *et al*.,2019; Anderson *etal*.,2011; Rahman, 2014). The commercial sterility testing procedure is a time consuming and laborious process that takes a minimum of 15 days to complete. The 15-dayduration is a time period during which the product is kept in abeyance from being released into the market and hence a form of economic burden to the manufacturer (Ghosal,2018;Varghese *et al*.,2014). Besides this there are other problems of traditional sterility testing method, which include

- **Destruction of Product**: Traditional sterility tests often require the destruction of the tested product, leading to waste and additional costs.
- **Variability of Microorganisms**: Not all microorganisms behave the same way. Some might be more resistant to thermal or chemical treatments, and others might be harder to detect or grow in laboratory conditions.
- **Heat Shock**: If the product isn't properly cooled before testing, thermophilic bacteria might undergo heat shock and not grow during the test, resulting in a false-negative.
- **Complex Food Matrices**: Certain food properties, like high fat content or viscosity, can interfere with the recovery and detection of microorganisms.
- **Limitations of Alternative Methods**: While rapid sterility testing methods are being developed, they might have limitations in terms of accuracy, sensitivity, or applicability to diverse food types.
- **Regulatory and Standards Compliance**: Meeting the varying sterility standards and guidelines set by different countries and organizations can be challenging, especially for products intended for international markets.
- **Evolving Strains**: As microorganisms evolve, new strains can emerge that might behave

differently during processing or testing, requiring continuous updates to testing protocols.

- **Equipment and Training**: Maintaining state-of-the-art equipment and ensuring that personnel are well-trained and updated on the latest methodologies is a continuous challenge.

This possesses a problem in ensuring safe and contamination free product without having to waste time and resources. This research study is for realising an alternative solution to detecting contamination.

Among the contaminating microbes whether spoilage causing or pathogenic microbes, interactions within communities are inevitable. In food ecosystems, several forms of interactions have been examined, including antagonistic and coordinated behaviour, as well as interactions in which the development or metabolism of one organism is favoured by the growth of another (Gram *et al*.,2002). These interactions are vital for the microbial communities for their sustenance. Many bacterial species interact with one another in a population-dependent manner to control their physiological processes via a mechanism known as Quorum Sensing (QS) (Frederix & Downie 2011). Through QS, bacteria are able to gauge their population density and accordingly regulate the expression of certain genes, often those involved in collective behaviors such as bioluminescence, biofilm formation, and the production of virulence factors (Liuet *al*.,2013; Paluch *et al*.,2020; Chen *et al*.,2002).

Quorum sensing is a mechanism by which bacteria communicate and coordinate their behavior based on their population density. This communication is mediated by chemical signal molecules called autoinducers (Miller & Bassler,2001). One such autoinducer is the N-acyl homoserine lactones (AHLs) that have been primarily reported in Gram-negative bacteria (Declerck*et al*.,2007). As individual bacterial cells grow and divide, they produce and release small amounts of these specific signalling molecules, known as the auto-inducers, into the surrounding environment. When the bacterial population is sparse, these molecules diffuse away, and their concentration remains low. As the population density of the bacteria increases, the concentration of the auto-inducer molecules also rises. Once the concentration reaches a threshold level (indicating a sufficient number or "quorum" of bacteria is present), the bacteria can detect this change. Upon sensing a critical concentration of autoinducers, the bacteria undergo a coordinated change in gene expression across the population (Beenker*et al*.,2022; Papenfort & Bassler,2016; Nguyen *et al*.,2019). This global shift enables the

community of bacteria to express specific genes simultaneously. If auto-inducers like AHLs are released by contaminating, quorum sensing microbial populace, can they be used as a biomarker indicative of microbial contamination?

Quorum sensing (QS) is a cell-to-cell communication mechanism utilized by bacteria to coordinate gene expression in response to fluctuations in cell-population density. This communication process relies on the production, release, detection, and response to auto-inducers, the extracellular signalling molecules. As the bacterial population grows, the concentration of these signalling molecules increases. Once a threshold concentration is reached, the signalling molecules can activate or repress target genes that govern various physiological activities (Asad and Opal,2008; Muller *et al*., 2006; Sturme*et al*.,2002).

2.4 Functions and Role of Quorum Sensing in a Bacterial Population

Quorum sensing provides a mechanism for bacteria to gain advantages in environments where resources are limited, and competition is fierce. It's a form of social cooperation, ensuring that certain behaviors (like producing virulence factors or forming a biofilm) are carried out in a coordinated fashion, which increases their overall effectiveness.

Table 7. Mechanisms of Quorum Sensing in Bacterial Behavior and Adaptation

Function	**Mechanism**	**Reference**
Biofilm formation	QS regulates the formation of biofilms, which are communities of bacteria adhered to surfaces surrounded by an extracellular matrix. Biofilms enhance bacterial resistance to antibiotics and the host immune response.	Costerton*et al*., 1999
Virulence Factor Production	QS can control the expression of virulence genes. Instead of expressing virulence factors all the time (which can be energetically costly), bacteria coordinate their attack once a critical mass is reached.	Rutherford &Bassler, 2012.
Sporulation	In some bacterial species, QS signals can help coordinate the formation of spores, a form of bacterial dormancy that helps the bacteria withstand unfavorable environmental conditions.	Pastor, 2011.
Competitive Advantages	QS can give bacterial populations a competitive advantage in mixed microbial communities by enabling them to adapt more efficiently to changes in their environment.	Ng &Bassler., 2009.
Bioluminescence	Some marine bacteria produce and emit light in a QS-dependent manner. This luminescence is often thought to aid in avoiding predation or in mutualistic interactions with marine animals.	Waters&Bassler.,2005.
Antibiotic	Some bacteria regulate the production of antibiotics	Straight&Kolter., 2009.

Production	via QS, ensuring that antibiotic synthesis only occurs when it's most effective.	

2.5 Relevance of Quorum Sensing in contamination

Quorum sensing is a critical factor in bacterial contamination events in various environments. Strategies that can interfere with or inhibit QS represent a promising avenue for controlling contamination and managing bacterial infections (Kumari *et al*.,2016; Maddela *et al*.,2019; Zhang *et al*.,2018) QS has a 3-fold role and relevance in contamination.

a. QS plays a vital role in contamination, especially in environments such as food products, medical devices, and water systems. The ability of bacterial pathogens to communicate and coordinate their actions through QS can exacerbate contamination events. The biofilm formation on contact surfaces for instance, wherein QS plays a major role, can act as a reservoir for ongoing contamination (Davies *et al*., 1998).

b. In case of food spoilage and contamination, post contamination, QS can proliferation and propagation of the contaminant (spoilage causing & pathogenic) and can also regulate the production of spoilage factors, such as enzymes, toxins and other metabolites, making food unsafe to eat or causing it to deteriorate more quickly (Skandamis & Nychas., 2012). In some bacterial pathogens, virulence factors are controlled by QS. These virulence factors can include toxins, enzymes, and other molecules that cause damage to a host or give the bacteria a competitive advantage (Rutherford & Bassler., 2012).QS that was once associated only with microbial activity and disease causing ability is now recognised as a key factor in contamination (Borea *et al*., 2018; Zhang *et al.,* 2018.)

c. QS enables bacteria to coordinate their activity and adapt to changing environmental conditions which might have serious consequences for contamination events. For instance, QS plays a role in conferring increased resistance to disinfectants and antibiotics by mediating the production of protective extracellular polymers and other resistance mechanisms. (Mah& O'Toole.,2001.)

Expression of QS is therefore integral in a microbial niche, including those in the food matrices and autoinducers such as the AHL common to all microbial ecosystems.

2.6 Quorum Sensing : Signalling Molecules

Quorum sensing (QS) systems rely on signalling molecules for communication. Depending on the bacterial species and the specifics of the system, different classes of signalling molecules can be involved. These signalling molecules allow bacteria to monitor their population density and adjust their gene expression accordingly. The understanding of these molecules and their receptors offers potential therapeutic avenues, such as quorum sensing inhibitors, to combat bacterial pathogenicity and biofilm formation. (Dickschat, 2010; Whiteheadet *al*., 2021; Whiteley*et al*., 2017).

Table 8. Shows Types of Quorum Sensing Molecules, Their Producers, structures and Examples

Molecule Type	Produced by	Structure/Characteristic	Example Organisms	References
AHLs (N-Acyl Homoserine Lactones)	Gram-negative bacteria	Homoserine lactone ring attached to a fatty acyl chain	*Vibrio fischeri, Pseudomonas aeruginosa, Agrobacterium tumefaciens*	Waters &Bassler., 2005
AIPs (Autoinducing Peptides)	Gram-positive bacteria	Short peptide signals	*Staphylococcus aureus, Streptococcus pneumoniae, Bacillus* species	Thoendel M, *et al.* 2011kk
I-2 (Furanosyl Borate Diesters)	Both Gram-negative and Gram-positive bacteria	Derived from the spontaneous rearrangement of DPD	*Escherichia coli, Vibrio harveyi, Salmonella typhimurium*	Chen X, *et al*., 2002
Cis-2-Unsaturated Fatty Acids	Gram-negative bacteria	Fatty acid-based molecules	*Pseudomonas aeruginosa*	Schuster & Greenberg., 2006
DSF (Diffusible Signal Factor)	Gram-negative bacteria	Typically based on cis-11-methyl-2-dodecenoic acid	*Xanthomonas campestris*	Ryan & Dow., 2008
PQS (Pseudomonas Quinolone Signal)	*Pseudomonas aeruginosa*	Quinolone-based signaling molecule	*Pseudomonas aeruginosa*	Pesci *et al*., 1999

Among the listed quorum sensing (QS) molecules, N-Acyl Homoserine Lactones (AHLs) are the most common and often considered the most relevant. While all the QS signaling molecules have their importance and relevance in specific contexts, AHLs stand out because of their broad distribution, significant role in pathogenesis, and extensive research attention. AHLs are produced by a wide range of bacteria, making them a common signalling molecule across diverse bacterial species. Some of the most studied bacterial pathogens, use AHLs in their QS systems to regulate virulence factors, biofilm

formation, and antibiotic resistance. The critical role of AHLs in regulating bacterial behaviors that are relevant to human health, such as biofilm formation and virulence, has made them a target for potential therapeutic interventions. Compounds that can interfere with AHL production or reception are being studied as potential anti-virulence therapies (Rasko& Sperandio.,2010).

2.7 AHL, QS molecule of the Gram Negative bacteria

AHLs are the most prevalent type of autoinducer, with about 10% of proteobacteria isolated from different environments (Tan *et al*.,2015) containing AHL. *Vibrio fischeri* was the first AHL (Eberhard *et al*., 1981), and later research has shown other AHLs produced by different Gram-negative proteobacteria. These include N-butyryl-homoserine lactone (C4-HSL), N-(3-hydroxybutyryl)-homoserine lactone (3-OH-C4-HSL), N-hexanoyl-homoserine lactone (C6-HSL), N-(3-oxodecanoyl)-homoserine lactone (3-oxo-C10-HSL), and others (Valienteet *al*., 2009; Wang *et al*., 2013; Tan *et al*., 2014; Jamuna & Ravishankar, 2016) AHLs plays a essential role in bacterial communication and coordination. LuxI and LuxR are two essential components. AHLs have diverse structures due to variations in saturation, substitutions and acyl chain moiety (Acet*etal*.,2021 & kawaguchie *et al*.,2008) that gets into three categories:

- Simple acyl,
- 3-hydroxyacyl, and
- 3-oxoacyl groups

The structure and concentration of AHL are both extremely important. The length of the acyl chain typically ranges from 4 to 12 carbons, with the first 2 carbons increasing to C4, C6, and C8 (Huang *et al*. 2016), which highlights the chemical compounds and general structure of lactones. A lactone ring is made up of amide and carbonyl groups as well as one hydrocarbon chain. Although N-acyl homoserine lactone signal molecules are found in gram-negative bacteria, oligopeptides are found in gram-positive bacteria (Kumari *et al*. 2008; Swift *et al*., 1994 & Safari *et al*., 2014). The generic structure of AHL is shown in Figure-1.0

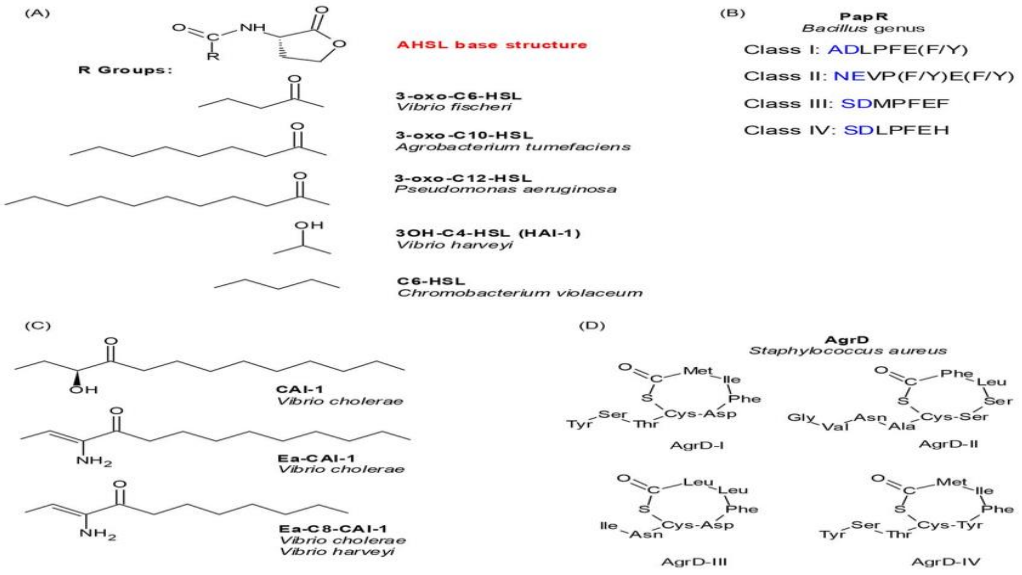

Fig 2. Shows specificity and complexity in bacterial quorum sensing
(Source:https://www.researchgate.net/publication/304618691_Specificity_and_complexity_in_bacterial_quorum-sensing_systems

N-Acyl Homoserine Lactones (AHLs) are a class of signaling molecules used predominantly by Gram-negative bacteria in the communication process of quorum sensing (QS), giving bacterial cells the ability to gauge their population density and accordingly regulate the expression of certain genes. AHLs consist of a homoserine lactone ring and a variable acyl side chain. The nature and length of this side chain, which can vary, provides specificity to the AHL and, therefore, specificity to the quorum sensing communication.

Mechanism of action of AHL

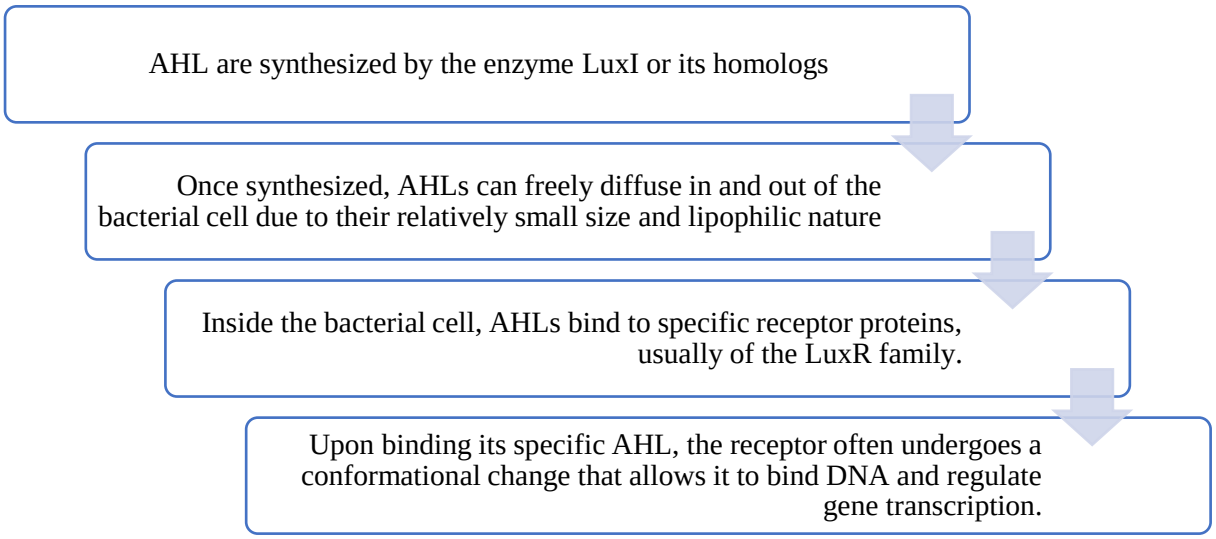
AHL are synthesized by the enzyme LuxI or its homologs
Once synthesized, AHLs can freely diffuse in and out of the bacterial cell due to their relatively small size and lipophilic nature
Inside the bacterial cell, AHLs bind to specific receptor proteins, usually of the LuxR family.
Upon binding its specific AHL, the receptor often undergoes a conformational change that allows it to bind DNA and regulate gene transcription.

Fig 3. Mechanism of AHL

The genes controlled by these complexes are often those involved in collective behaviours such as biofilm formation, virulence factor production, bioluminescence, and more.

At low bacterial cell densities, AHL concentrations in the environment are low, and thus intracellular AHL concentrations are insufficient to induce a response. However, as the bacterial population grows and produces more AHLs, the concentration of AHLs in the environment (and within bacterial cells) reaches a threshold level (Sharif *et al*.,2008). This threshold concentration is enough to bind and activate the LuxR-type receptors, thereby initiating the transcription of quorum-sensing regulated genes (Brameyer&Heermann,2015). Some bacteria also produce enzymes that can degrade AHLs (Dong & Zhang, 2005; Fetzner,2015). This is another level of regulation that can modulate quorum sensing responses or interfere with the quorum sensing of other bacteria.

2.8 Analytical methods for AHL screening

Detecting N-Acyl Homoserine Lactones (AHLs) in various matrices requires sophisticated analytical methods as shown in the table below.

Table 8. Shows Analytical methods for quorum sensing molecule detection and quantification

Analytical Method	Matrices	Brief Description	Reference
Biosensor Assays	Liquids (water, bacterial cultures)	Use of AHL-sensitive bacterial strains that produce a detectable response (e.g., luminescence, pigment) to AHL presence.	Winson, M. K., *et al*,.1998.
Thin Layer Chromatography (TLC)	Liquids (bacterial cultures)	Separation of AHLs on TLC plates, followed by overlay with AHL-sensitive bacterial strains to detect active spots.	Shaw, P. D., *et al*., 1997.
High Performance Liquid Chromatography (HPLC)	Liquids (water, bacterial cultures)	Separation and quantification of AHLs. Sometimes combined with mass spectrometry (HPLC-MS) for enhanced specificity.	Ortori, C. A., *et al*., 2011.
Mass Spectrometry (MS)	Various (soil, water, bacterial cultures)	Direct identification and quantification of AHLs based on mass-to-charge ratio. Often used in tandem with other methods like HPLC.	Schuster, M., *et al*., 2003.
Nuclear Magnetic Resonance (NMR)	Liquids (bacterial cultures)	Identification and structural elucidation of AHLs based on magnetic properties of atomic nuclei.	Yin, W. F., *et al*., 2012.
Fluorescence-based Assays	Liquids (water, bacterial cultures)	Use of fluorescent tags or compounds that fluoresce upon interaction with AHLs.	Fekete, A., *et al*., 2010.
Capillary Electrophoresis (CE)	Liquids(bacterial cultures)	Separation of AHLs based on their charge and size in an electric field.	Ling, J. L., *et al*.,2015.

Analyzing AHLs is pivotal in understanding quorum sensing and there have been recently developed methods for analyzing AHL in different matrices. Horacek*etal* (2022) identified 18 AHLs using a comprehensive chiral analytical procedure that included solid phase extraction, Gas Chromatography with Mass Spectrometry (GC-MS/MS), and Liquid Chromatography with Mass Spectrometry (LC-MS/MS) methods. Notably, LC-MS/MS outperformed GC-MS/MS in sensitivity and indicated enantioselectivity differences based on AHL size and hydrophobicity. Lepine*et al*. (2018)& Honda *et al* (2019) investigated AHLs in biological and Wastewater samples using high-performance liquid chromatography coupled with mass spectrometry and Fourier transform mass spectrometry, respectively, bringing light on AHL diversity in different environments, particularly in wastewater systems with complex quorum sensing dynamics.

Bose *et al* (2016) used High-Performance Liquid Chromatography-tandemn Mass Spectrometry (HPLC-MS/MS) to assess acyl-homoserine lactones (AHLs) in *Vibrioaceae* bacteria. Their precise technique, which was optimized in a total of six steps, indicated extensive AHL quorum sensing signals among these bacteria. AHL profiles were comparable in closegenetic strains. This study not only shed light on the complexities of AHL synthesis within *Vibrionaceae,* but it also established a flexible detection approach applicable to a wide range of bacterial samples, including therapeutically relevant strains

A number of studies have used MALDI-TOF mass spectrometry to identify and quantify acyl homoserine lactones (AHLs). Kim *et al*(2015) developed a MALDI-based 3-oxo AHL quantification technique that uses Girard's reagent T (GT) to improve sensitivity and decrease the limit of detection (LOD) and limit of quantitation (LOQ) in *Psesudomonas* bacterial culture (Panchavinin *et al.*,2019) employed Fourier-transform mass spectrometry (FTMS) to identify distinct AHL species in activated sludge samples from wastewater treatment facilities. Wang *et al* (2014) used gas chromatography-mass spectrometry (GC-MS) to create a technique for identifying AHLs in soil solutions.

Ghani *et al* (2014) used MALDI-TOF to describe the breakdown of AHLs by a bacteria isolated from sea water and verified the lactonase-based mechanism of action. Honda *et al* (2019) investigated LC-FTMS analysis being more sensitive method than TLC Bioassays to detect AHL in waste water treatment plants.

2.9 AHL in food matrices

N-Acyl Homoserine Lactones (AHLs) play pivotal roles in the quorum sensing of many Gram-negative bacteria, regulating various behaviours such as virulence, biofilm formation, and antibiotic resistance (Hossain *et al*.,2015). Their presence in food matrices can be indicative of bacterial contamination, and hence detecting them can be useful for assessing food safety(Liu et al.,2006;Bruhn *et al*.,2004).AHLs in food matrices can arise from the growth of spoilage bacteria or pathogenic bacteria that employ quorum sensing(Bai and Ravi, 2016). Detection of AHLs in food can provide an early indication of microbial contamination even before visible spoilage occurs (Nychas*et al*.,2018; Gram *et al*.,2002). The possible methods for detection of AHL food matrices could be as shown below: -

2.11 Analytical Methods for AHL Detection in Food Matrices:

- Biosensor Assays: AHL-sensitive bacterial strains can be employed. These strains produce a detectable response (e.g., luminescence) in the presence of AHLs. (Morin *et al*., 2013)
- High Performance Liquid Chromatography (HPLC): Often combined with mass spectrometry (HPLC-MS) to detect and quantify AHLs in food matrices with high specificity. (Ortori*et al*., 2007)
- Thin Layer Chromatography (TLC): After extraction from food matrices, AHLs can be separated on TLC plates. An overlay with AHL-sensitive bacterial strains can then identify active AHL spots. (Cataldi,2004)
- Nuclear Magnetic Resonance (NMR): Suitable for identification and structural elucidation of AHLs from food extracts. (Stachel,1985)

However, detecting AHLs in food matrices can be challenging due to the complexity of the matrix. AHLs can bind with food components or degrade during processing and storage, which might lead to false negatives. Efficient extraction methods, often including acidification, solvent extraction, or solid-phase extraction, are needed to isolate AHLs from food matrices before analysis.

The research problem in hand therefore is to explore the possibility of using AHL as a biomarker, indicative of bacterial contamination in food, in particular the Gram-negative bacteria. Most pathogens causing foodborne illness are gram-negative in nature.

3.0 OBJECTIVES

1. To validate and standardize AHL detection and estimation using test organisms in culture media.
2. To concentrate, detect and estimate Acyl Homoserine Lactone in Water and Food matrix
3. To compare various techniques to determine the optimal method for validation and detection of Acyl Homoserine Lactone
4. To explore the possibility of developing a Lateral Flow assay using Acyl Homoserine Lactone as the target molecule.

4.0 METHODOLOGY / PLAN OF STUDY

4.1 Plan of study

Table 9. Plan of Study

1st to 6th month	**AHL spiking studies in culture media**			
7th to 12th month		**AHL detection in spiked culture media**		
13th to 18th month			**AHL detection in water spiked with test organisms**	
19th to 24thmonth				**AHL detection in**

		foods spiked with test organisms	
25th to 30th month			**Data consolidation and thesis writing**

1. Detect and estimate spiked AHL in culture media.
2. Detect and estimate AHL in sterile Culture media and quantify the microbial load as CFU/ml
3. Detect and estimate AHL in culture media spiked with Test Organism -1 and quantify the microbial load as CFU/ml
4. Detect and estimate AHL in culture media spiked with Test Organism -2 and quantify the microbial load as CFU/ml
5. Detect and estimate AHL in culture media spiked with Test organisms -1 & 2 and quantify the microbial load as CFU/ml
6. Detect and estimate AHL in sterile Low-acid foods and quantify the microbial load as CFU/ml
7. Detect and estimate AHL in Low-acid foods spiked with Test Organism -1 and quantify the microbial load as CFU/ml
8. Detect and estimate AHL in Low-acid foods spiked with Test Organism -2 and quantify the microbial load as CFU/ml
9. Detect and estimate AHL in Low-acid foods spiked with Test organisms -1 &2 and quantify the microbial load as CFU/ml

4.2Materials & Methods

Materials

1. Culture
   a. Test organisms
      i. Pseudomonas fluorescens

ii. *Serratia marcescens*

2. Culture Media
   i. Pseudomonas Agar
   ii. Serratia Agar
   iii. Soyabean Casein Digest Agar
   iv. Soyabean Casein Digest Medium
3. Acyl Homoserine Lactone standards
4. Dimethyl Sulphoxide (DMSO)
5. 0.22 um Filter syringe
6. SPE C18 Cartridge

Methods

Table 10. Methodology

S.no	Experiment	Methodology
1	Culture procurement	Standard National and International Repositories Cultures of *Serratia marcescens and Pseudomonas fluorescens*will be purchased from the National Collection of Industrial Microbes (NCIM) in Pune, India, and Agricultural Research Services (ARS) Northern Regional Research Laboratory (NRRL) of the United States Department of Agriculture (USDA) in Peoria, USA. The purchase will depend on the availability of these cultures at these repositories at the time of order.
2	Culture stock preparation and maintenance	Glycerol stock, Slant, Plate (Dutez*et al*.,2000)
3	AHL stock preparation and maintenance	Standards will be purchased from Sigma Aldrich, India Prepare AHL stock by dissolving the compound in DMSO (10 to 500mM) concentration and store in -20 ºC(Churchill*et al*.,2022)
4	Microbial load estimation	Culture plating method (in CFU/ml)
5	AHL Concentration in Water	Water sample filteredsyringe(0.22 µm)pore size will be used and concentrate AHLs by using SPE C18 cartridge followed by nitrogen stream, rotary evaporation or ethyl acetate extraction.(Yates *et al*.,2002)
6	AHL Concentration in food	**Sample Preparation and Separation of AHL**: First, blend the food sample thoroughly to ensure even distribution of AHL Add ethyl acetate to the blended sample. Vortex the mixture for 5-10 minutes. After vortexing, centrifuge the mixture at 6000 rpm for 10 minutes. Carefully filter the

		supernatant through a 0.22 µm syringe filter. **Concentraion of Extracted AHL:**Concentrate the extracted AHL by using a rotary evaporator to evaporate the solvent. Finally, purify the concentrated AHL by passing it through a C18 SPE cartridge and eluting the AHL with methanol (laj*et* al.,2022).
7	AHL estimation and detection	HPLC-High-performance liquid chromatography LC-MS-Liquid chromatography mass spectrometer UPLC-HRMS-Ultra pressure liquid chromatography-high resolution mass spectrometer.
8	AHL molecule targeted Lateral flow essay	Wollner *et al*.,2010

5.0 RATIONALE TO THE STUDY

Acyl Homoserine Lactone (AHL) are quorum sensing molecules that are secreted by bacterial communities. These molecules contribute to several physiological role towards sustenance of a microbial population and therefore are common to all microbial ecosystems including that of food and water and hence could be traced in environments with bacterial population. Can AHL be used as a bio-marker to detect bacterial contamination ? If so this can eliminate the need for time-consuming and labour intensive culture methods.

6.0EXPECTED OUTCOMES

AHLs as biomarkers will provide a specific and reliable indicator of bacterial contamination, it will enhance the overall safety and quality control measures within the food processing practices.

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